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Original Paper

Comparative screening of cadmium stress responses among three rice cultivars

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Abstract

Plants are frequently confronted with heavy metal stress, which is one of the major environmental concerns in agriculture. Cadmium is a potent toxic and hazardous soil contaminant that is introduced into the atmosphere by industrial pollution. It can impose detrimental effects on plant growth, crop productivity and quality. To evaluate Cd-induced physio-biochemical stress response, a comparative study was conducted with three rice (*Oryza sativa* L.) cultivars ('Maharaj', 'Pratiksha' and 'Khitish') treated with 10 µM $CdCl_2$. Cytotoxicity assessments were performed to determine cell death rates. The study confirmed that cv. 'Maharaj' was the most Cd stress tolerant due to high proline, phytochelatin, and antioxidant concentration, resulting in lower cell death rate. Cv. 'Pratiksha' was moderately tolerant while cv. 'Khitish' was more susceptible to Cd stress, showing drastic reduction in cysteine and sugar concentration, and Hill activity, with noticeable increase of lipid peroxidation, methylglyoxal and starch concentration.

Key words: antioxidant, cadmium, reactive oxygen species, rice, secondary metabolites, stress.
Abbreviations: CAT, catalase; Cd, cadmium; GPX, guaiacol peroxidase; GR, glutathione reductase; HMs, heavy metals; MDA, malondialdehyde; MG, methylglyoxal; NPSH, non-protein thiols; PAL, phenylalanine ammonia lyase; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase.

Introduction

With the rapid urbanization and industrial development there has been an exponential increase in the emission of toxic heavy metals in the atmosphere, which has caused serious long-term threats to living organisms (Dar et al. 2020). Heavy metal toxicity is one of the major abiotic stresses causing health hazards in animals and plants through entering food chain (Maksymiec 2007). Being non-biodegradable (Purakayastha, Chhonkar 2010) and highly reactive they can directly disrupt ultrastructural, biochemical and molecular processes of plants culminating in stunted growth, leaf chlorosis, impaired seed germination, lipid peroxidation, gene mutation and photosynthetic abnormality leading to early senescence in plants (Gill 2014). Heavy metals such as cadmium, chromium, lead and mercury are mainly generated by weathering of minerals, but anthropogenic origin of Cd, including electroplating, sludge dumping, mining, smelting, industrial discharge, utilization of pesticides and phosphate fertilizers, is a major concern as a threat to agricultural land (Kubier et al. 2019). Cadmium ranks 7th in the list of top 20 toxic metals with a concentration of ~0.1 mg kg^{-1} in the Earth's crust (Hussain et al. 2020). Unpolluted soil may contain Cd below 0.5 mg kg^{-1}, but it can reach up to 3.0 mg kg^{-1} depending on soil composition (Zhang et al. 2021).

Rice (*Oryza sativa* L.) is one of the three main growing food crops and India has been the major contributor of rice to the global food supply (Fahad et al. 2019). Since 1970, West Bengal has been known as region with high rice diversity – more than 5000 rice varieties (Sinha, Mishra 2013). These cultivars vary not only in their yield parameters, but also in abiotic stress tolerance potential, especially towards heavy metal contamination depending on their metal uptake capacity. Absorption and bioaccumulation of Cd are entirely age-, dosage- and genotype-dependent among various rice cultivars (Fahad et al. 2019). Many soil parameters like pH, organic matter, presence of other ions, types of crop plants and their root exudates affect the bioavailability of Cd (Sarwar et al. 2010). Cd can be easily mobilized from root cortical tissue to the xylem through formation of complexes with organic acids, triggering Cd accumulation into the aerial parts of crop plants. Plants do not possess any Cd specific transporter but it is aided by metal transporter families like NRAMP (Natural Resistance Associated Macrophage Protein), ZIP (ZRT-IRT like protein), ABC transporter, P-type ATPase, CAX, YSL (Yellow Strip-Like) and LCT transporters (Paul et al. 2022).

Being a non-redox metal, Cd cannot produce reactive oxygen species (ROS) directly, but it interferes in electron transport chains creating oxidative stress in plants, which can be detoxified by upregulating enzymatic antioxidants (superoxide dismutase, catalase, guaicol peroxidase and

glutathione reductase) and nonenzymatic antioxidant molecules to serve as cellular redox buffers (Barman et al. 2020). For estimation of Cd toxicity, various parameters can be assessed, including morphological (root, shoot length, vigour index), physiological (chlorophyll concentration, Hill activity, lipid peroxidation, which are direct indicators of damage), followed by enzymatic and nonenzymatic (phytochelatin, phenolics, secondary metabolites) antioxidative defence mechanisms. Cd accumulation in rice causes lipid peroxidation and damages phospholipids via disruption of membrane architecture, mainly acting on polyunsaturated fatty acids (Banerjee, Roychoudhury 2018). It has been well documented in rice that Cd toxicity is directly linked to plant growth impairment, chlorosis, alteration of antioxidant status, cell death, disruption of mesophyll cells, Fe scarcity, and disrupted photosynthesis through thylakoid stacking (Rizwan et al. 2016; Arif et al. 2019).

The objective of present work was comparative screening of Cd stress mediated effect (at morphological, physiological, biochemical and cytotoxicity levels) on three locally grown rice cultivars of West Bengal ('Maharaj', 'Pratiksha' and 'Khitish').

Materials and methods

Plant material

Seeds of three local rice cultivars 'Maharaj', 'Pratiksha' and 'Khitish' were obtained from the Chinsurah Rice Research Station, West Bengal and surface sterilized with 0.2% dithane solution (anti-fungal agent) for 15 min. The seeds were rinsed thoroughly with deionized water to remove residual surface sterilizer and soaked overnight in the dark (about 16 h).

Hydroponic growth medium

To estimate all parameters, the plants were grown and maintained in hydroponic solution. For germination, batches of 25 disinfected seeds were kept on separate Petri plates (90 mm) with moist filter paper at 30 °C for 72 h in the dark. Then, germinated seedlings were transferred to a beaker containing ½ Hoagland's mineral nutrient solution and allowed to grow at light intensity with photon flux density of 260 µmol m^{-2} s^{-1}, photoperiod 16 h, 70% relative humidity and 30 °C for seven days. Seedlings were treated with 10 µM $CdCl_2$. Seedlings maintained in Hoagland solution without Cd treatment were considered as a control. The seedlings were harvested 7 days after the start of the treatment and washed with distilled water to remove traces of salt residues. In preliminary trials seedlings were exposed to 10 and 12 µM $CdCl_2$, but the higher concentration was too toxic all seedlings showed chlorosis before 7 days of cultivation. Therefore, only treatment 10 µM was used. Each treatment including the control set was performed in triplicate.

Measurement of physiological parameters

At 14 days growth of seedlings, root and shoot length, and dry weights of each plant were measured. Relative water content (RWC) was calculated using the formula of Barrs and Weatherley (1962). The vigour index was estimated using the formula of Abdul-Baki and Anderson (1973):

Vigour Index = Germination % × Seedling Length (Root + Shoot).

Determination of photosynthesis-related parameters

For determination of chlorophyll concentration, finely chopped fresh leaf tissue (50 mg) was immersed in 2 mL 80% acetone overnight and the content of chlorophyll *a*, chlorophyll *b* and carotenoids was determined spectrophotometrically by recording absorbance at three wavelengths 470, 646.8 and 663.2 nm, respectively. The concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids was calculated as described by Lichtenthaler (1987).

Hill activity was estimated by the method of Bauer and Senser (1979). About 100 mg of leaves were crushed in 0.05 M sucrose-phosphate buffer followed by centrifugation. The pellet was suspended in sucrose phosphate buffer. To this chloroplast suspension, 0.05 M sucrose phosphate buffer and 0.03% 2,6-dichlorophenolindophenol was added and initial absorbance was taken at 610 nm. The reaction mixture was kept under sunlight for 20 min and absorbance was measured again at 610 nm. The difference in optical density was calculated and the activity was expressed as µmol 2,6-dichlorophenolindophenol reduced per g chlorophyll h^{-1}.

Assessment of stress markers

For proline analysis, 100 mg of fresh plant tissue was crushed in 1 mL of 0.1 M sulfosalicylic acid and centrifuged in the cold. Then, 0.5 mL supernatant and 1 mL glacial acetic acid with 1 mL ninhydrin solution were mixed and incubated for 1 h at 100 °C, followed by immediate ice cooling. Then, 2 mL of toluene was added and mixed thoroughly. Absorbance of the upper aqueous phase was measured at 520 nm and proline concentration was estimated by comparison with a standard curve (Bates et al. 1973).

For estimation of lipid peroxidation, the concentration of thiobarbituric acid-reactive substances (malondialdehyde, MDA) was measured. Fresh plant tissue (100 mg) was homogenized in 1 mL of 0.1% trichloroacetic acid and centrifuged. Supernatant (500 µL) was mixed with 2 mL of 20% trichloroacetic acid and 0.5% thiobarbituric acid mixture and kept for 30 min in a boiling water bath. Optical density of the resultant sample was recorded at 532 nm using a spectrophotometer (Heath and Packer 1968). MDA concentration was expressed as µmol g^{-1} FW tissue.

Generation of hydrogen peroxide (H_2O_2) was measured following the method of Loreto and Velikova (2001). Fresh 500 mg shoot and root samples were extracted with 3 mL

of 1% trichloroacetic acid followed by centrifugation in the cold. The reaction mixture contained 0.75 mL of 10 mM phosphate buffer, 1.5 mL of 1 M freshly prepared potassium iodide and 0.75 mL plant extract. The absorbance of the reaction solution was measured at 390 nm.

The concentration of methylglyoxal was estimated according to the method of Yadav et al. (2005). Fresh 0.5 g of plant tissue was extracted in 3 mL of 0.5 M perchloric acid with grinding in a pestle and mortar. The sample was kept on ice for 15 min and centrifuged in the cold. The coloured supernatant was decolorized by incubating with charcoal for 15 min at room temperature, followed by centrifugation at 11 000 rpm for 10 min to eliminate charcoal. The sample was then neutralized in a saturated solution of potassium carbonate at room temperature for 15 min and further centrifuged. The methylglyoxal reaction was prepared by mixing in order 250 µL of 7.2 mM 1,2-diaminobenzene, 100 µL of 5 M perchloric acid and 650 µL of the neutralized supernatant. The absorbance of the methylglyoxal was monitored at 335 nm.

Enzymatic antioxidants

Crude tissue extract was prepared by crushing 1 g fresh plant tissue in 10 mL of 0.2 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA, followed by and centrifugation. The resultant supernatant was stored at –20 °C to be used for estimation of all antioxidative enzymes following Elavarthi and Martin (2010).

To assay superoxide dismutase (SOD) activity,100 µL supernatant was added to 2.1 mL reaction mixture containing 80 mM Tris buffer (pH 8.9), 0.12 mM EDTA, 0.003% bovine serum albumin, 10.8 mM tetramethylethylenediamine, 600 µM riboflavin in 5 mM KOH, and 6 mM nitroblue tetrazolium. The mixture was placed 30 cm below a light source for 2 min and the absorbance was measured at 560 nm. A solution with nitroblue tetrazolium but without enzyme and light served as a blank. Unit SOD activity was calculated as 50% decrease in photoreduction of nitroblue tetrazolium per mg protein min^{-1}.

To assay catalase (CAT) activity, the reaction mixture contained 980 µL of 50 mM phosphate buffer (pH 7), 20 mM H_2O_2 and 20 µL supernatant. The disappearance of H_2O_2 was recorded by measuring the absorbance of solution at 240 nm continuously for 1 min. Enzyme activity was calculated using the extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1}).

To assay guaiacol peroxidase (GPX) activity, a reaction mixture was prepared by adding 980 µL of 50 mM phosphate buffer, 50 mM guaiacol, 10 mM H_2O_2 and 20 µL supernatant. H_2O_2-dependent oxidation of guaiacol was estimated by an increase in absorbance at 470 nm and monitored for 1 min. Enzyme activity was calculated using the extinction coefficient – 26.6 mM^{-1} cm^{-1}.

To assay glutathione reductase (GR) activity, the assay system consisted of 980 µL of 50 mM phosphate buffer, 0.75 mM 5,5'-dithio-bis-2-nitrobenzoic acid, 0.1 mM NADPH,1 mM oxidized glutathione and 20 µL supernatant. Change in absorbance at 412 nm was checked for 1 min. Enzyme activity was calculated using the extinction coefficient of the product 2-nitro-5-thiobenzoate (14.15 M^{-1} cm^{-1}).

The total protein concentration from 500 mg leaf samples was estimated according to Lowry et al. (1951).

Non-enzymatic antioxidants

Non-protein thiol (NPSH) concentration was determined according to Cakmak and Marschner (1992). About 500 mg of fresh leaf tissue was homogenized in 5 mL of 5% meta-phosphoric acid and centrifuged at 15 000 rpm for 15 min. To the following extract, 2.5 mL 150 mM phosphate buffer (pH 7.4) with 5 mM EDTA and 0.5 mL of 6 mM 5-5-dithiobios-2-benzoic acid was mixed. The mixture was incubated at room temperature and absorbance was read at 412 nm. Phytochelatin concentration was estimated by subtracting the values of reduce glutathione from NPSH values (Bhargava et al. 2005).

The total phenolic concentration of tissue was determined using Folin-Ciocalteu reagent with gallic acid as a standard. Fresh 500 mg tissue was homogenized in 80% ethanol and centrifuged. Supernatant (200 µL) was mixed with 800 µL Folin-Ciocalteu reagent followed by addition of 2 mL of sodium carbonate. The resulting mixture was incubated for 30 in the dark condition and absorbance was measured at 765 nm (Su et al. 2007).

Total flavonoid concentration was quantified according to Lamaison and Carnet (1990). At first, ethanolic extract of the plant tissue was mixed with 10% aluminium chloride solution (1:1, v/v) and kept at room temperature for 30 min. The intensity of resultant yellow colour sample was estimated at 415 nm.

Total cysteine concentration was determined spectrophotometrically using the method of Roy Choudhury et al. (2007). Ice-cold 5% (v/v) perchloric acid was used to homogenize fresh 0.5 g of shoot followed by cold centrifugation. The supernatant was treated with acid ninhydrin reagent and absorbance of the extract was measured at 560 nm.

Ascorbic acid concentration was determined spectrophotometrically, using the method of Herrig et al. (2002). Fresh 100 mg plant tissue was crushed in 1.5 mL of trichloroacetic acid and centrifuged. The reaction mixture consisted of 0.3 mL supernatant, 0.2 mL Folin phenol reagent and 1.5 mL deionized H_2O. Absorbance was measured at 760 nm.

Estimation of total soluble sugar and starch content

Total soluble sugar concentration was assayed by a previously described method (Dubois et al. 1956). Fresh 1 g plant tissue was homogenized in 80% aqueous ethanol for 15 min in a water bath, followed by centrifugation of the extract at 2000 rpm for 20 min. Then, 1 mL supernatant was mixed thoroughly with phenol-sulphuric acid reagent and absorbance of the solution was estimated at 490 nm.

Starch was measured by the method of McCready et al. (1950). The end residue of the centrifugation produced during total soluble sugar extraction was suspended in 2.5 mL with distilled water and 52% perchloric acid. Then, the mixture was centrifuged for 20 min at 2000 rpm. Final volume of resultant supernatant was made to 100 mL. Starch concentration in the filtrate was quantified in terms of glucose and a 0.9 factor was used for converting glucose to starch concentration.

Phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase (PAL) activity was estimated following the protocol of Dickerson et al. (1984). Plant samples were homogenized in 3 mL of 0.1 M sodium borate buffer (pH 7.0) containing 0.1 g insoluble polyvinylpyrolidone. The homogenate was centrifuged for 20 min at 15 000 g. The reaction mixture consisted of 0.4 mL supernatant, 12 mM L-phenylalanine, and 0.5 mL of 0.1 M borate buffer (pH 8). The reaction mixture was incubated for 30 min at 30 °C and PAL activity was measured at 290 nm.

Salicylic acid concentration

Salicylic acid (SA) concentration was measured according to Warrier et al. (2013). About 100 mg fresh plant tissue was ground to powder and 1 mL of acetone was added. The sample was then centrifuged and 500 µL of the supernatant was added to 0.1% freshly prepared ferric chloride to make a total volume of 3 mL. The absorbance of the solution was then measured at 540 nm with a spectrophotometer.

Cell viability assay

Cell viability assay was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the method of Carmichael et al. (1987) to evaluate cell viability of plant roots. Fresh 10 mg root tissue was placed in a microcentrifuge tube containing 1.5 mL of MTT solution and incubated in the dark for 4 h. The root samples were chopped and added to 0.5 ml KOH (2 N) and 0.5 mL 99.99% dimethyl sulfoxide solution in 2-mL microcentrifuge tubes. The tubes were centrifuged at 1000 g for 5 min to sediment the root pieces completely. Then, absorbance of the supernatant was recorded at 570 nm.

Measurement of Cd

Rice roots and shoots were dried in an oven at 80 °C for 48 h. The oven-dried samples were ground into fine powder. About 0.1 g of the ground samples were weighed and digested with a tri-acid mixture of HNO_3 : HCl : $HClO_4$ and the resultant solution volume was adjusted to 25 mL with deionized water. The concentration of Cd was analysed by using inductively coupled plasma-optical emission spectroscopy (ARCOS, Spectro, Germany). The translocation factor was estimated as the ratio between metal concentration in shoot and root (Takarina et al. 2017).

Statistical analysis

Graphs were prepared using Graphpad Prism 8. Experimental data were statistically analyzed using two-way analysis of variance (ANOVA) test followed by Sidak multiple comparison tests. A heat-map was generated for visual interpretation of Cd tolerance level in the tested cultivars. The SPSS 23 software package was used to conduct all statistical analyses.

Results

Cadmium concentration

Cd was taken up in aerial parts of 14-day-old treated rice seedlings of all three cultivars. Under 10 µM $CdCl_2$ treatment,'Maharaj' contained 13.5 and 5.8 mg Cd kg^{-1} DW in roots and shoots, respectively. Roots and shoots of 'Pratiksha' contained 12.9 mg and 3.3 mg Cd. The lowest accumulation occurred in 'Khitish' roots (11.8 mg kg^{-1}) and shoots (1.7 mg kg^{-1}) (Table 1). Maximum Cd translocation from roots to shoots was in 'Maharaj' (Transfer factor = 0.425), while 'Pratiksha' (Transfer factor = 0.252) and 'Khitish' (Transfer factor = 0.142) showed lower Cd translocation.

Effect of Cd on plant growth, relative water content and vigour index

In both 'Maharaj' and 'Pratiksha' seedlings treated with Cd, significant differences in both root and shoot length did not occur, compared to control plants. Significant reduction (14%) was observed in Cd-treated 'Khitish' shoots (Fig. 1A).

Table 1. Assessment of Cd content in root and shoot of three *Oryza sativa* cultivars. Data presented are mean values ± SD for three replicates. Different letters indicate statistically significant differences ($P < 0.05$). ND, not determined

Cultivar	Treatment	Cd concentration (mg kg^{-1})		Transfer factor (shoot/root)
		Shoot	Root	
'Maharaj'	Control	ND	ND	ND
	10 µM Cd	5.745 ± 0.035 a	13.500 ± 0.003 a	0.425 ± 0.020 a
'Pratiksha'	Control	ND	ND	ND
	10 µM Cd	3.255 ± 0.007 b	12.870 ± 0.176 b	0.252 ± 0.150 b
'Khitish'	Control	ND	ND	ND
	10 µM Cd	1.675 ± 0.177 c	11.750 ± 0.020 c	0.142 ± 0.040 c

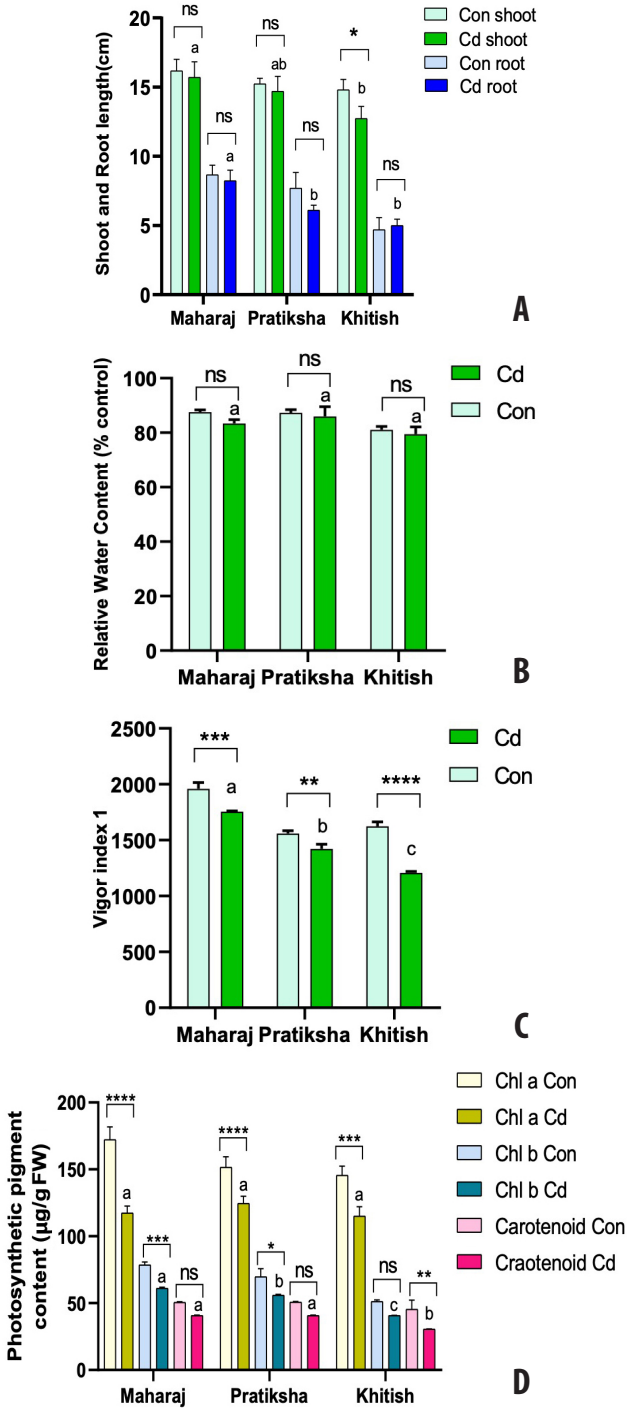

Fig. 1. Effect of Cd on growth (A), relative water content (B), vigour index (C), photosthetic pigment concentration (D) of *Oryza sativa* cultivars. Error bars represent standard deviation obtained from the mean data of three individual replicates. Bars followed by (*), (**), (***) and (****) show significant difference at $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ level, respectively, by using *t* test.

Decline in biomass (Table 2) and relative water content in all Cd-treated cultivars was noted, but the differences were not statistically significant (Fig. 1B). Furthermore, vigour index, which reflects optimum growth conditions of plants, was significantly lower in Maharaj' (9%), 'Pratiksha' (11%), 'Khitish' (26%) seedlings treated with Cd (Fig 1C).

Table 2. Effect of Cd on dry biomass of three *Oryza sativa* cultivars. Data presented are mean values ± SD for three replicates. Different letters indicate statistically significant differences ($P < 0.05$)

Cultivar	Plant biomass (g)	
	Control	Cd (10 µM)
'Maharaj'	0.023 ± 0.031 a	0.019 ± 0.002 a
'Pratiksha'	0.024 ± 0.001 a	0.016 ± 0.030 ab
'Khitish'	0.019 ± 0.002 a	0.014 ± 0.017 b

Effect of Cd on photosynthetic parameters

The most prominent effects of Cd treatment were decreased photosynthetic parameters in all Cd-treated rice seedlings. The decrease of photosynthetic pigment concentration in Cd stressed seedlings was 19, 19 and 32% for chlorophyll *a*, 19, 23 and 21% for chlorophyll *b*, and 19, 21 and 32% for carotenoids in 'Maharaj', 'Pratiksha' and 'Khitish' cultivars, respectively (Fig. 1D). Hill activity of treated seedlings was downregulated under Cd toxicity, compared to control plants. Significant decline in Hill activity of 'Khitish' (27%) was evident (Fig. 2A).

Effect of Cd on stress indicators

Cd treatment caused significant increase in MDA concentration of 'Khitish' roots (61%) and shoots (50%) followed by roots (19%) and shoots (43%) of 'Pratiksha' (Fig. 2B). Proline, an important osmolyte that has a positive correlation with stress condition, showed increased concentration in Cd-treated shoots of 'Maharaj' (32.85%) and 'Pratiksha' (8.88%) (Fig. 2C). Plants under stress tend to accumulate a considerable amount of methylglyoxal – in Cd-treated 'Khitish' seedlings, there was a significant increase in methylglyoxal production (shoot 72%, root 94%), while only roots of Cd-treated 'Pratiksha' seedlings had significantly higher (75%) methylglyoxal concentration (Fig. 2D). The endogenous H_2O_2 level showed differential response in the three cultivars under Cd stress. In treated 'Maharaj' seedlings, the increase in endogenous H_2O_2 in roots (36%) and shoots (42%)was not as high as in shoots of 'Pratiksha' (58%) and 'Khitish' (58%) seedlings, which showed significant increase in endogenous H_2O_2 concentration (Fig. 2E).

Effect of Cd on antioxidative enzyme activity and antioxidants

Various antioxidant enzymes such as SOD, GPX, GR and CAT act as a first line of defence against oxidative injury. In Cd-treated 'Maharaj' seedlings, SOD activity increased both in shoots (98%) and roots (35%), compared to control seedlings. There was a statistically significant increase in the activity of CAT, GPX and GR (32, 37 and 20% in shoots and 15, 56 and 18% in roots) in Cd-treated 'Maharaj' seedlings. In 'Pratiksha seedlings, significant increase of activity

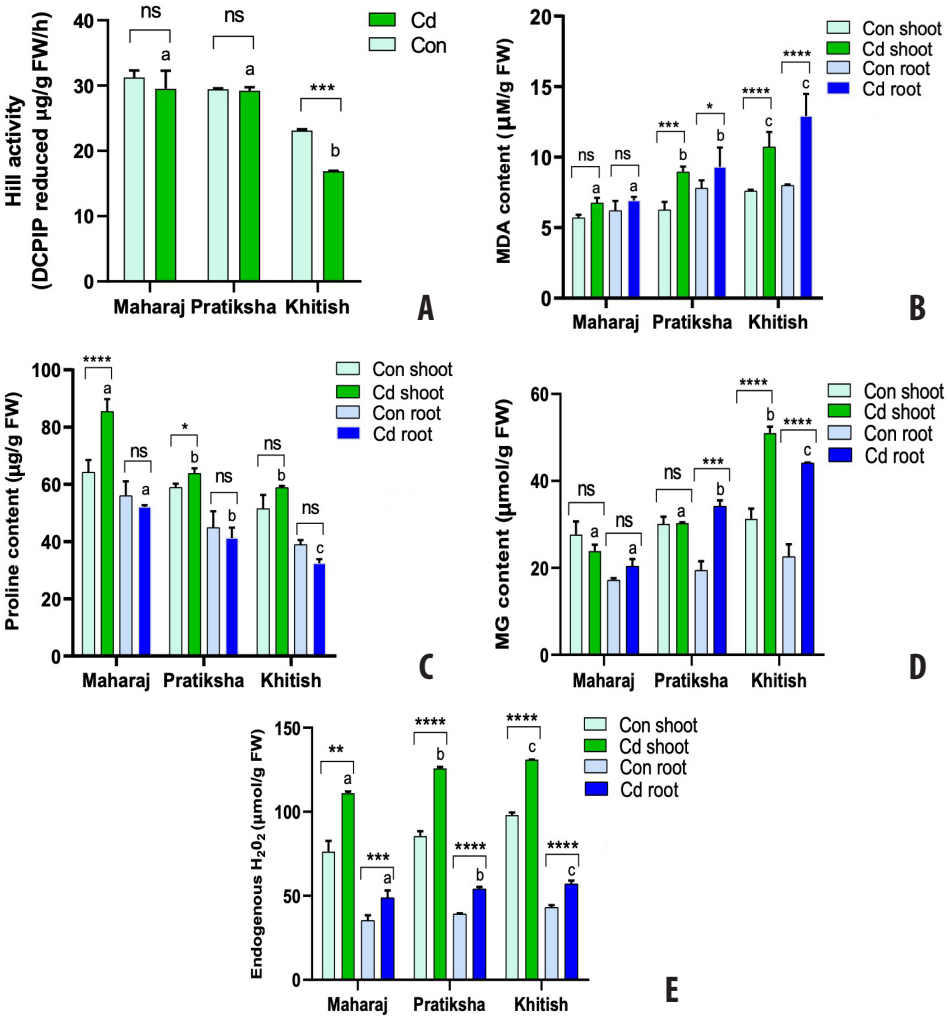

Fig. 2. Effect of Cd on Hill activity (A), MDA concentration (B), proline concentration (C), methylglyoxal concentration (D), and concentration (E) of *Oryza sativa* cultivars. Error bars represent standard deviation obtained from the mean data of three individual replicates. Bars followed by (*), (**), (***) and (****) show significant difference at $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ level, respectively, by using *t* test.

of all enzymes (SOD, CAT, GPX and GR) was observed in shoots (30, 20, 31 and 14%, respectively). In contrast, 'Khitish' seedlings did not exhibit any significant difference in antioxidative enzymatic activity, excepting GPX activity in shoots (14%) and roots (23%) of Cd-treated seedlings (Fig. 3A, B, C, D).

NPSH concentration in Cd-treated 'Khitish' seedlings did not increase significantly, while treated shoots of 'Maharaj' seedlings showed statistically significant increase of NPSH concentration in shoots (55%) and roots (18%) (Fig. 4A). Phytochelatin concentration was significantly higher in shoots and roots of Cd-treated 'Maharaj' (78 and 40%) and 'Pratiksha'(25 and 13%) seedlings, but the effect was less pronounced in Cd-treated 'Khitish' seedlings (Fig. 4B). Total protein concentration was significantly lower in 'Khitish' (24%) and 'Pratiksha' (24%) seedlings in comparison to the control, but not in 'Maharaj' seedlings (Fig. 4C).

Significant increase of phenolic concentration in roots (43%) and shoots (55%) of 'Pratiksha' and roots (21%) and shoots (18%) of 'Maharaj' seedlings was observed (Fig. 4D). In comparison to control plants, flavonoid concentration was elevated significantly both in roots (26 and 22%) and shoots (80 and 79%) of treated 'Maharaj' and 'Pratiksha' seedlings, respectively. Significant decrease in flavonoid concentration (31%) was evident in roots of 'Khitish' seedlings (Fig. 4E).

Cd exposure led to a significant increase in accumulation of cysteine in roots (81 and 25%) and shoots (70 and 19%) of 'Maharaj' and 'Pratiksha' seedlings, respectively. In roots of Cd-treated 'Khitish' seedlings, cysteine concentration did not increase significantly (Fig. 4F). Cd exposure resulted in a significant increase of total sugar concentration both in roots (22%) and shoots (15%) of 'Maharaj' seedlings, compared to the control. However, in Cd-treated 'Khitish' seedlings, total sugar concentration was close to the control level (Fig. 5A). Significant elevation of starch concentration was recorded in shoots (25%) and roots (17%) of 'Khitish' seedlings (Fig. 5B).

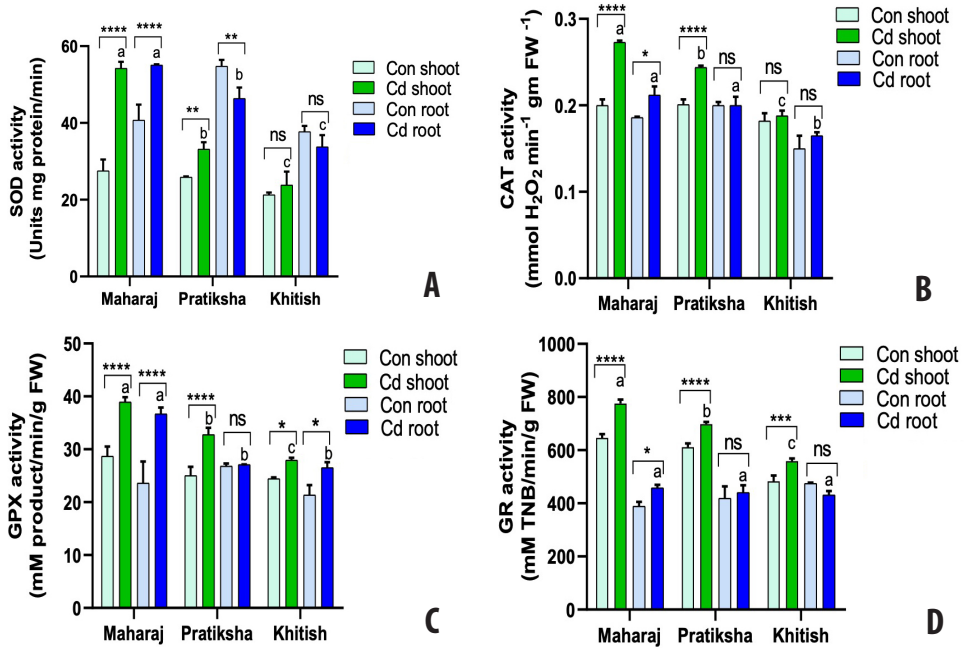

Fig. 3. Effect of Cd on superoxide dismutase activity (A), catalase activity (B), guaiacol peroxidase activity (C), and glutathione reductase activity (D) of *Oryza sativa* cultivars. Error bars represent standard deviation obtained from the mean data of three individual replicates. Bars followed by (*), (**), (***) and (****) show significant difference at $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ level, respectively, by using *t* test.

Effect of Cd on PAL, ascorbic acid and salicylic acid

In 'Maharaj' and 'Pratiksha', PAL activity increased by 9.57% and 3.44% in shoots and by 10.4% and 11.5% in roots, respectively. In 'Khitish' seedlings treated with Cd, PAL activity was decreased by about 5% in shoots (Fig. 5C). In comparison to control plants, no considerable changes in ascorbic acid concentration were found in 'Khitish' and 'Pratiksha' seedlings, but it increased in shoots of Cd-treated 'Maharaj' seedlings (6%) (Fig. 5D). In 'Maharaj' and 'Pratiksha' seedlings treated with Cd, significant increase of salicylic acid concentration was evident in shoots (25 and 15%, respectively) in comparison to control seedlings. In Cd-treated 'Khitish' seedlings, salicylic acid concentration was similar to or less than the control values (Fig. 5E).

Effect of Cd on cell viability

Among the three cultivars, Cd-treated 'Khitish' seedlings had the highest rate of cell death in roots, while 'Maharaj' seedlings showed the lowest cell death rate (Fig. 5F).

A clustered heat map was created with differential colour intensity for visual perception of the experimental data for all considered parameters (Fig. 6). Multivariate analysis generated a three component model explaining variance (Appendix 1, Appendix 2). The loadings of PC1 for proline, cysteine, sugar, and antioxidants formed a group with negative loads, whereas starch, MDA, methylglyoxal formed a group with positive loadings.

Discussion

Determination of morphophysiological and biochemical responses of Cd accumulator plants is crucial as they may have a cascading effect in Cd-inflicted injury, resulting in overall growth and yield decrement in paddy fields.

Cadmium accumulation

The highest Cd concentration occurred in roots and shoots of 'Maharaj' seedlings and the lowest in 'Khitish' seedlings, while 'Pratiksha' seedlings had an intermediate metal concentration. The largest amount of Cd was in roots in all three cultivars, which might be due to direct contact of roots with Cd. The translocation factor showed that the 'Maharaj' cultivar is a Cd accumulator – to a lesser extent for the other two cultivars (Table 1) (Hidayati, Rini 2020).

Effect of Cd on morphophysiological responses

Cd-mediated growth inhibition was more pronounced in roots of treated rice seedlings due to their direct exposure to Cd in growth medium. This caused impairment of many physiological processes including cell division, elongation in root tip, water imbalance and nutrient deficiency (Zhao et al. 2019). Growth reduction was also reported in wheat (Abbas et al. 2017) and spinach (Younis et al. 2016). A gradual decrease in relative water content results in loss of turgor potential leading to stomatal closure and reduced photosynthetic rates under Cd stress. Our results revealed

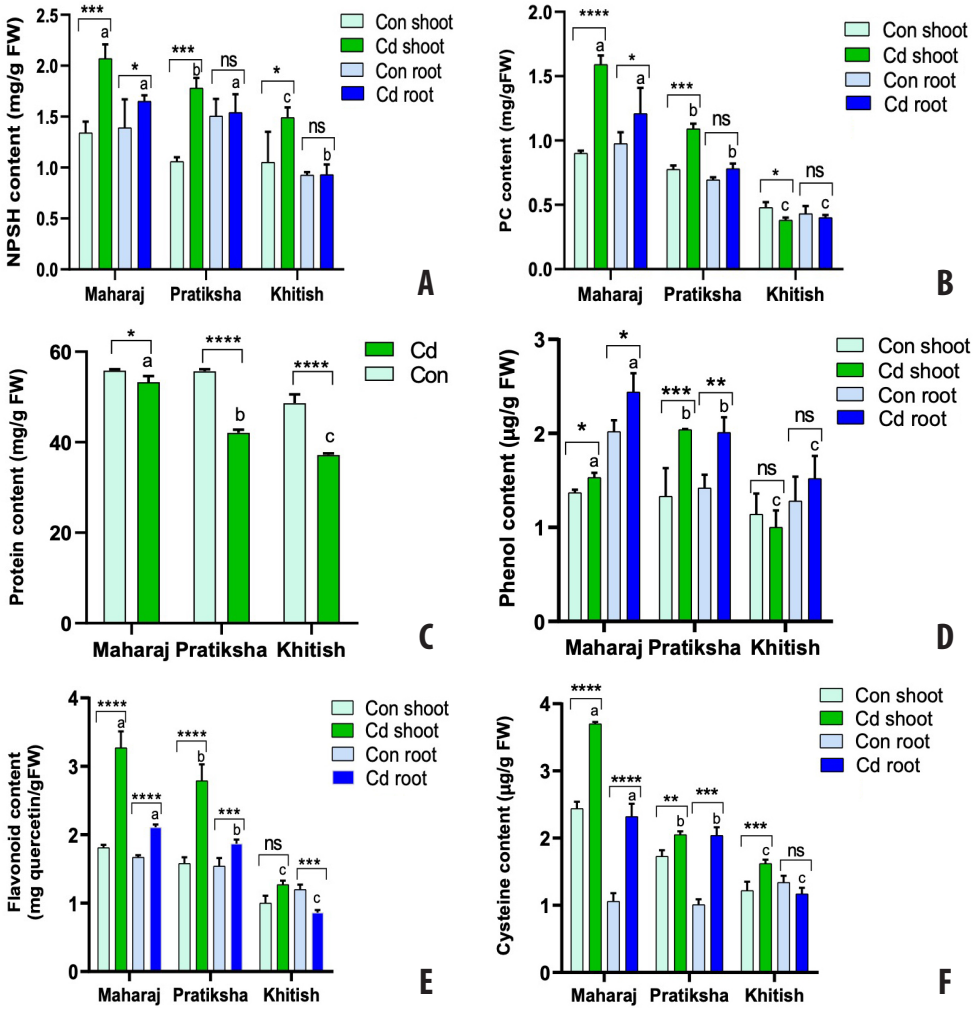

Fig. 4. Effect of Cd on non-protein thiol concentration (A), phytochelatin concentration (B), protein concentration (C), phenolic concentration (D), flavonoid concentration (E), cysteine concentration (F) of *Oryza sativa* cultivars. Error bars represent standard deviation obtained from the mean data of three individual replicates. Bars followed by (*), (**), (***) and (****) show significant difference at $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ level, respectively, by using *t* test.

slightly higher relative water content in 'Maharaj' seedlings, indicating Cd tolerance compared to the Cd sensitive cultivars, which is in good accordance with reports of several workers (Kang et al. 2012; Sun et al. 2015). Cd-treated 'Khitish' seedlings displayed maximum decline in vigour index, which might indicate poor growth status (Paul et al. 2022). Cd-induced decline in chlorophyll *a*, chlorophyll *b* and carotenoid concentration reflects compromised photosynthesis, which might be associated with repression of porphobilinogen synthesis leading to subsequent decrease in activity of aminolevulinic acid dehydratase manifested as chlorosis (Majumder et al. 2020). A sharp decline in photosynthetic parameters of 'Khitish' seedlings indicates that this cultivar is more susceptible to Cd stress in comparison to 'Maharaj' and 'Pratiksha'. Moreover, decreased Hill activity of 'Khitish' seedlings indicates disruption of electron transport in photosystem II and the oxygen-evolving complex causing inactivation of ADP phosphorylation, resulting in decreased CO_2 assimilation and altered net photosynthesis leading to compromised growth (Yang et al. 2009).

Effect of Cd on stress markers

In plants, heavy metal toxicity results in overproduction of ROS, leading to oxidative damage of cellular macromolecules and biological membranes. The most mutagenic by-product of lipid peroxidation is MDA, which has been utilized as a biomarker to measure the degree of membrane damage. The observed increased production of MDA in 'Khitish' seedlings indicates Cd susceptibility due to extensive membrane damage (Khanna-Chopra et al. 2019). Proline concentration has positive correlation with tolerance under metal stress, which can mitigate membrane damage, since proline reacts with phospholipids, scavenge ROS and impedes oxidative stress. Besides functioning as an osmolyte, proline serves as metal chelator, free radical

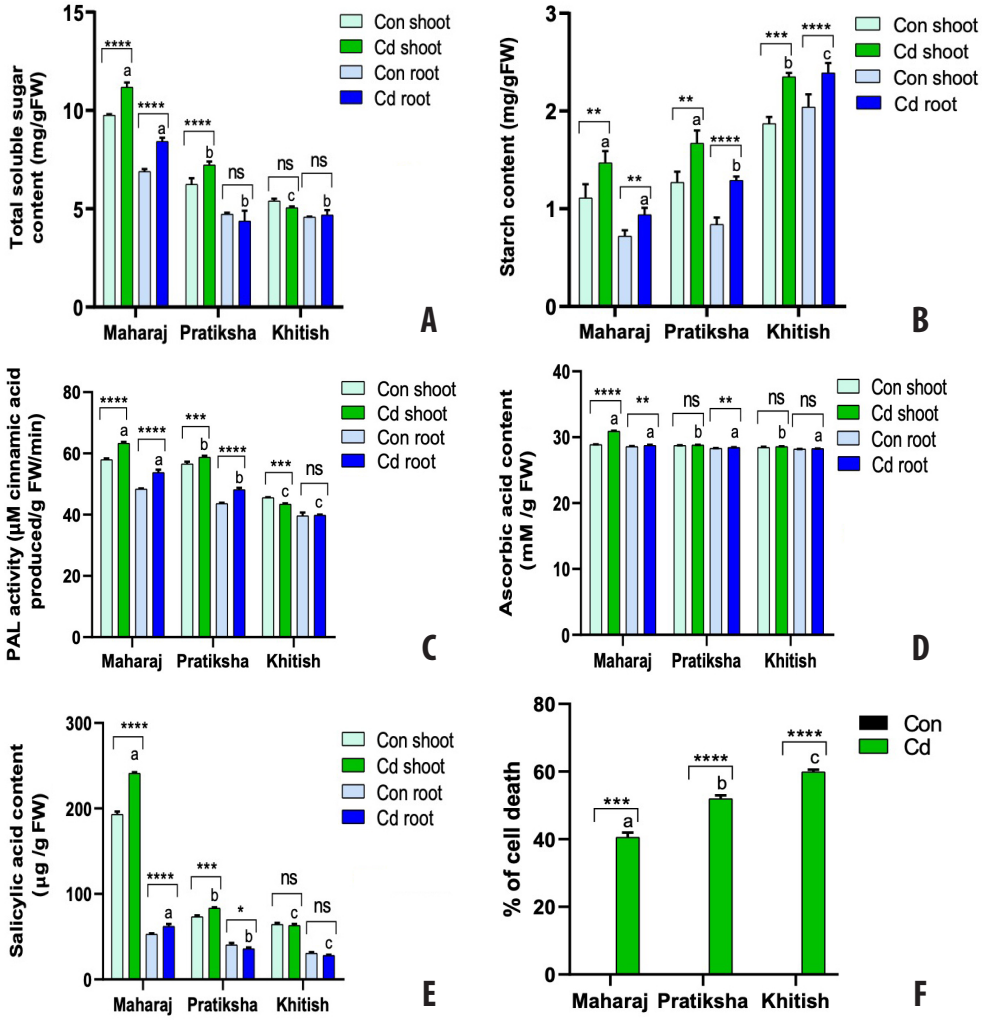

Fig. 5. Effect of Cd on total soluble sugar concentration (A), starch concentration (B), phenylalanine ammonia lyase activity (C), ascorbic acid concentration (D), salicylic acid concentration (E), cell death rate (F) of *Oryza sativa* cultivars. Error bars represent standard deviation obtained from the mean data of three individual replicates. Bars followed by (*), (**), (***) and (****) show significant difference at $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ level, respectively, by using *t* test.

scavenger, signalling molecule, helps in stabilization of membranes and prevents protein degradation (Saadati et al. 2019). Similarly, accumulation of free proline was reported in rice under Cd toxicity (Barman et al. 2020). Cd exposure increased proline concentration in 'Maharaj' seedlings more than in 'Khitish'. It is possible that the former showed better tolerance to Cd through stabilization of membranes and averting oxidative burst by eliminating excess ROS. Thus, higher proline concentration indicates better adaptability to environmental stress (Hidangmayum et al. 2019).

Plants facing stress accumulate toxic aldehydes of which methylglyoxal is the most ubiquitous (Hoque et al. 2016). Metal toxicity-induced methylglyoxal accumulation causes detrimental effects either by direct reaction with cellular components like lipids, sugars and DNA or by promoting oxidative damage by higher ROS production. Increased methylglyoxal levels were previously reported in rice plants under excessive Cd and Cu stress (Mostofa et al. 2015). Also, rice, mustard, tobacco and millet accumulated two to six fold higher amounts of methylglyoxal in response to salinity, drought and cold stresses (Yadav et al. 2005). Reaction of superoxide dismutase with excessive amount of ROS yields H_2O_2 under stress. Higher H_2O_2 concentration in 'Khitish' caused raised levels of ROS generation in Cd sensitive plants (Srivastava et al. 2014).

Effect of Cd on antioxidative system

To combat metal load, plants have developed well organized defence mechanisms to detoxify the free radicals and prevent cellular and molecular level damage. In such cases, plants possess low molecular weight antioxidant enzymes such as SOD, GR and CAT that eliminate the ROS and protect plant parts from cytotoxic damage (Agarwal et al. 2010). Our experiments showed that enzymatic antioxidant activity was most increased in 'Maharaj' and 'Pratiksha',

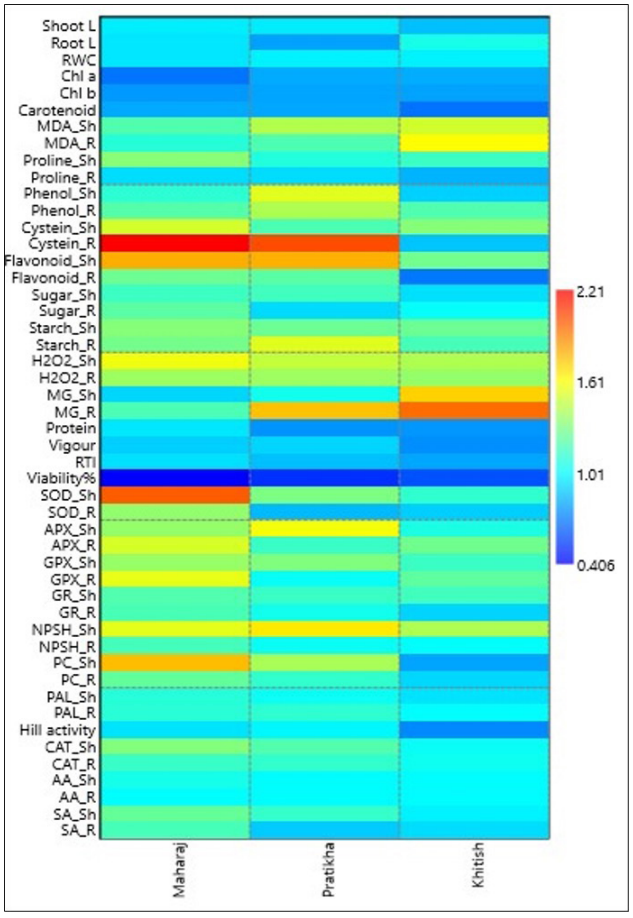

Fig. 6. Heat map showing fold change in physiological and biochemical parameters of Cd treated *Oryza sativa* cultivars with respect to control.

compared to the control. SOD catalyzes dismutation of superoxide radical to produce H_2O_2 while CAT and GPX serves as major detoxifying enzymes and detoxify H_2O_2 produced by SOD activity through degradation of H_2O_2 into water and molecular oxygen (Stephenie et al. 2020, Anjum et al. 2016). Considerable promotion of enzymatic activity in 'Maharaj' compared to 'Khitish' and 'Pratiksha' indicates lower accumulation of H_2O_2, and more efficient tolerance to Cd toxicity. Low SOD activity in 'Khitish' may result in inactivation of antioxidative enzymes under stress suggesting poor tolerance to Cd. It is known that Cd stress-enhanced enzymatic activity can minimize ROS accumulation resulting in greater stress tolerance in plants (Guo et al. 2019; Hakeem et al. 2019).

Detoxification of Cd is also achieved by non protein thiol groups. Concomitant induction of NPSH levels in 'Maharaj' seedlings with Cd treated plants indicates the active participation of NPSH in Cd detoxification. This is in good accordance with the study of Mishra et al (2014), where prominent elevation of NPSH in Cd-treated *Withania somnifera* followed by Cd chelation was observed. In response to environmental stress, plants efficiently produce S-rich protein, phytochelatins by phytochelatin synthase, which can form complexes with heavy metal ions like Cd, Zn, Cu and As. Greater accumulation of phytochelatins in 'Maharaj' and 'Pratiksha' seedlings might be due to oligomerization of reduced glutathione to form phytochelatins during Cd exposure, which is involved in the chelation and sequestering Cd into vacuole. In contrast, low phytochelatin concentration in 'Khitish' seedlings may be related to low reduced glutathione production limiting phytochelatin synthesis resulting in Cd sensitivity (Lin, Aarts 2012). Cysteine imparts its protective role against Cd toxicity by forming of non-protein thiols that are involved in Cd mitigation. Being the precursor of metal chelator phytochelatins, reduced glutathione, non-protein thiol, has high potential to provide tolerance to plants by Cd sequestration in the vacuole (Roy et al. 2016). Cysteine provides antioxidative protection by forming non-protein thiols against Cd contamination in plant. Cys is the rate-limiting factor for synthesis of reduced glutathione, which is a low-molecular weight, non-protein thiol molecule that can mitigate Cd-induced oxidative stress in plants (Guha et al. 2020). High cysteine concentration was noted in two cultivars, which can serve as potent alleviators of Cd stress, as well as efficient ROS scavenging providing better tolerance.

To determine ROS detoxification capacity of the cultivars we estimated concentration of potential non enzymatic antioxidants such as phenolics, flavonoid compounds, which act as chelators of metal ions and can reduce lipid peroxidation by scavenging the lipid alkoxyl radical (Granato et al. 2018). It is well documented that for detoxification of ROS plants utilize phenolics and flavonoids as essential non enzymatic antioxidants. Higher phenolic concentration of 'Maharaj' and 'Khitish' seedlings due to higher PAL activity resulted in less damage. Similarly, 50 µM Cd treatment to rice increased PAL activity by 81% (Jali et al. 2019). Photosynthetically-produced carbohydrates sustain plant growth and act as osmoprotectants as well as membrane protectants, and also serve as a source of energy for cellular activity (Rosa et al. 2009). Heavy metal stress strongly hampers carbon metabolism resulting in poor nutritional status of plants. Cd-exposed 'Maharaj' seedlings had massive accumulation of total sugar content by prompt breakdown of starch, which acts not only as a free radical scavenger, but also promotes ROS metabolism employing the oxidative pentose phosphate pathway (Hu et al. 2012), reflecting highest capacity of osmotic adjustment and better Cd tolerance among other cultivars. Moreover, Cd exposed 'Khitish' seedlings exhibited lower accumulation of sugars along with huge accumulation of starch, possibly by less utilization or conversion into soluble sugar as a consequence of inhibition of net photosynthesis causing retarded growth and metabolism, and suggesting Cd susceptibility (Asgharipour et al. 2011).

Effect of Cd on secondary metabolites

Primary metabolites are involved in the synthesis of secondary metabolites through intermediate precursor molecules and these secondary metabolites play different roles in defence against stress. PAL is involved in phenylpropanoid synthesis and serves as a link between primary and secondary metabolism (García-Pérez et al. 2020). Cd-treated 'Maharaj' and 'Pratiksha' seedlings had higher PAL activity, indicating that phenolic compounds engaged in cell wall lignification from flavonoids precursor ensured enhanced protection against Cd stress. Previous studies also showed that stimulation of PAL during heavy metal stress accelerated secondary metabolite synthesis and enhanced oxidation resistance in *Hydrilla verticillata* for better adaptability to Cd (Kobyletska et al. 2022). However, PAL activity declined in 'Khitish' seedlings, which was correlated with poor defence capacity to stress (Zhang et al. 2020).

Plants synthesize salicylic acid either through cinnamic acid produced by the PAL pathway or from chorismate via isochorismate (Kovács et al. 2014). Being a ubiquitous plant signalling molecule, salicylic acid governs cellular redox status by production of monomerized NPR1 proteins that migrate from cytosol into the nucleus and accelerates activation of defence-related transcription factors like WRKY and TAG (Hao et al. 2012). The induction of salicylic acid accumulation in treated 'Maharaj' and 'Pratiksha' seedlings might be associated with improvement of photosynthesis, osmoregulation, and antioxidant defence system mechanisms to cope with oxidative injuries. This was also confirmed by the observation of Wang et al. (2020) that salicylic acid diminished Cd toxicity by lowering MDA and H_2O_2 accumulation and scavenging ROS, which promoted Cd stress tolerance potential in rice.

Effect of Cd on cell viability

Cell death negatively affects root metabolism and reduced uptake of nutrients and water from the soil, causing reduction of growth, biomass, photosynthetic pigments and plant yield (Guha et al. 2020). Treated 'Maharaj' plants showed upregulation of antioxidative defense system under Cd stress to cope with oxidative damage; in contrast, 'Khitish' seedlings exhibited steady increment of stress-related parameters, which is in agreement with the findings of the present work. This study is fully congruent to the idea that to withstand Cd stress, 'Maharaj' possessed some adaptive mechanism through production of high proline, PC, sugar, PAL, and SA. The Cd-sensitive 'Khitish' seedlings had higher MDA and MG concentration leading to ROS generation. 'Pratiksha' seedlings showed moderate adaptive mechanisms to manage Cd stress. The disparity of tolerance and sensitivity in rice seedlings in response to Cd stress is associated with a stress-mediated mechanism. Our study strongly supports previous work showing that 'Maharaj' seedlings are more Cd tolerant in every aspect compared to the 'Pratiksha' and 'Khitish' seedlings. Cadmium stress does not only cause environmental deterioration but also results risk to consumer health. Therefore, much more information is needed to provide deeper insight into the process of cadmium-induced stress minimization of rice varieties for better food security.

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Appendix 1. Values for loading of principal components during multivariate analysis of morpho-physiological parameters in rice varieties ('Maharaj', 'Pratiksha', 'Khitish')

Parameter	PC1	PC2	PC3
Shoot length	–0.39	–0.05	0.88
Root length	0.28	0.56	0.18
RWC	–0.31	–0.49	–0.50
Chlorophyll *a*	–0.39	–0.11	0.12
Chlorophyll *b*	–0.25	0.62	0.16
Carotenoids	–0.39	0.02	–0.06
Vigour	–0.39	–0.04	–0.38
Hill activity	–0.38	–0.21	0.08

Appendix 2. Values for loading of principal components during multivariate analysis of biochemical parameters in rice varieties ('Maharaj', 'Pratiksha', 'Khitish')

Parameter	PC1	PC2	PC3
MDA shoot	0.23	–0.07	–0.15
MDA rroot	0.21	0.15	–0.28
Proline shoot	–0.18	0.23	–0.14
Proline root	–0.21	–0.15	–0.01
Phenol shoot	–0.37	–0.01	0.14
Phenol root	–0.35	–0.15	–0.07
Cysteine shoot	–0.08	0.35	0.45
Cysteine root	–0.23	0.10	0.08
Soluble sugar shoot	–0.28	–0.20	–0.19
Soluble sugar root	–0.17	0.25	0.20
Starch shoot	0.20	–0.18	0.13
Starch root	0.01	–0.37	0.14
MG shoot	0.21	0.17	–0.15
MG root	0.23	–0.07	–0.09
SOD shoot	–0.23	0.08	–0.02
SOD root	–0.23	–0.06	–0.12
GPX shoot	–0.22	–0.13	0.29
GPX root	–0.18	0.24	0.31
GR shoot	–0.23	0.05	–0.01
GR root	–0.23	–0.04	0.17
CAT shoot	–0.23	–0.07	0.11
CAT root	–0.21	–0.16	0.15
PAL shoot	–0.23	–0.07	–0.04
PAL root	–0.20	–0.19	0.01
SA shoot	–0.22	–0.09	–0.11
SA root	–0.19	0.21	–0.14

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